The first 6′-*O*-sulfated phenylanthraquinones: isolation from *Bulbine frutescens*, structural elucidation, enantiomeric purity, and partial synthesis

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Received 28 April 2005; revised 15 June 2005; accepted 16 June 2005

Available online 12 July 2005

Dedicated to Professor W. Steglich and Professor E. Dagne, discoverers of the first phenylanthraquinone

Abstract—From the roots of *Bulbine frutescens*, the first sulfated phenylanthraquinones were isolated, together with their known sulfate-free analogs. Their structures were elucidated by spectroscopic and chiroptical methods, by acid hydrolysis or by partial synthesis. The new compounds have the usual stereo-orientation at the biaryl axis (i.e., with the acetyl portion above the anthraquinone plane) except for sodium *ent*-knipholone 6′-*O*-sulfate (and thus, also its hydrolysis product, *ent*-knipholone), which exhibit an opposite axial configuration. We also describe the first stereoanalysis of natural phenylanthraquinones, some of which were found to be not enantiomerically pure, some even near-racemic. We furthermore, report on the first X-ray structure analysis of a phenylanthraquinone, viz. 4′-*O*-demethylknipholone.

1. Introduction

Bulbine frutescens (L.) Wild (Asphodelaceae) is an ornamental plant widely grown in Botswana, while it is used for the treatment of various ailments, particularly for wound healing, in other Southern African countries.[1] Previous work on this plant yielded knipholone (**1**), 4′-*O*-demethylknipholone-4′-β-D-glucopyranoside (**2**), and gaboroquinones A and B,[2] of which compounds **1** and **2** showed remarkable antiplasmodial activities.[2,3] Other knipholone-related phenylanthraquinones with antiplasmodial activity have also been isolated from *B. capitata* and *B. abyssinica*,[3–6] making *Bulbine* species attractive plants for the search for further new metabolites.

We therefore, reinvestigated *B. frutescens*, especially with respect to the polar fractions as detected on TLC, which showed the presence of four unidentified metabolites. In this paper, we report on the isolation and structural elucidation of as yet unprecedented *O*-sulfated phenylanthraquinones, viz. compounds **5**–**8**, along with the corresponding non-sulfated 'parent compounds', **1**–**4**. The absolute configurations at the biaryl axes of the new sulfated phenylanthraquinones were established by CD spectroscopy, by comparison of both the sulfates and of their desulfated hydrolysis products with the authentic sulfate-free parent analogs, revealing that all these natural products have the same (preferential) absolute stereo-orientation—except for compound **5**: it has the constitution of an as yet unknown 6′-*O*-sulfate of knipholone, but its absolute configuration is opposite,[7] since its hydrolysis product is *ent*-knipholone (*ent*-**1**), not knipholone (**1**) itself. This, and the fact that the crystal structure of 4′-*O*-demethylknipholone (**3**), that is, the first X-ray structure analysis in this class of natural products, established that particular sample to be racemic, raised the question of enantiomeric purities of all these axially chiral biaryls. For this purpose, the first enantiomer analysis of phenylanthraquinones was developed, by chromatography on a chiral phase, showing that the biarylic compounds isolated here show largely divergent enantiomeric ratios.

Keywords: *Bulbine frutescens*; Sulfated phenylanthraquinones; Axial chirality; Enantiomeric resolution; LC–CD coupling; Crystal structure.
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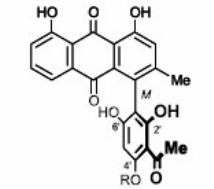
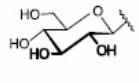
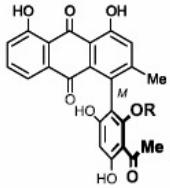
doi:10.1016/j.tet.2005.06.055

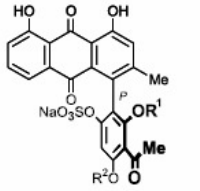


	R
1	Me
2	



	R
3	H
4	Me

5



	R'	R
6	H	
7	H	H
8	Me	H

2. Results and discussion

Flash chromatography of the organic extract (129 g) of the roots of *B. frutescens* followed by TLC comparison (SiO_2, $CHCl_3$/MeOH, 4:1) with authentic samples of phenylanthraquinones revealed the presence of four unidentified metabolites in the polar fractions (100% EtOAc to 20% MeOH in EtOAc), whilst TLC (SiO_2, $CHCl_3$/MeOH, 96:4) of the relatively nonpolar fractions (20% EtOAC to 50% EtOAc in petroleum ether) showed the presence of 4′-*O*-demethylknipholone (**3**) and isoknipholone (**4**), in addition to the previously isolated[2] compounds knipholone (**1**) and 4′-*O*-demethylknipholone-4′-β-D-glucopyranoside (**2**). Gel permeation chromatography of the polar fractions on Sephadex LH-20 ($CHCl_3$/MeOH, 1:1) followed by PTLC ($CHCl_3$/MeOH, 4:1) and repeated Sephadex LH-20 purification ($CHCl_3$/MeOH, 2:1) afforded four new compounds.

The EIMS of the first compound gave a molecular ion at *m/z* 434 corresponding to the molecular formula $C_{23}H_{16}O_8$ suggesting the presence of an isomer of knipholone (**1**). This was, at first sight, in agreement with the ^{1}H NMR spectrum of **1**, which showed three proton signals at δ 12.02, 12.52, and 13.87 characteristic of chelated hydroxyl groups, a three-proton ABC system (δ 7.53, dd, J=7.6, 1.1 Hz; 7.72, dd, J=8.2, 7.7 Hz; 7.27, dd, J=8.3, 1.2 Hz) typical of proton signals at positions 5-, 6-, and 7-, and proton signals at 7.27, 2.20, 2.67, and 3.98 characteristic of proton signals at H-2, C-3 methyl, acetyl methyl, and methoxy at C-4′, respectively. The above ^{1}H NMR spectral data closely resembled those of knipholone (**1**)[8] except that the signal characteristic of the proton at C-5′ in **1** (δ 6.24, s) was shifted downfield to δ 7.30.

An acetone solution of the compound, at room temperature, was transformed to knipholone within a few hours (according to NMR and by co-TLC with authentic sample), with the simultaneous liberation of a white precipitate, suggesting that the compound contained a readily hydrolyzable oxygen functional group. Possible substituents included an *O*-alkyl or an *O*-glycoside, but these functional groups were clearly ruled out since, they would be seen in both, the ^{1}H and in the ^{13}C NMR spectrum. Therefore, it had to be a heteroatom containing functionality such as an *O*-sulfate or an *O*-phosphate group. The white precipitate referred to above was found to contain the elements sodium and sulfur by EDX analysis (see Section 4 and Supporting information), hinting at the existence of a sulfate group, most probably as the sodium salt. The identity of the white solid as sodium sulfate was confirmed by standard tests (white precipitate with aqueous $BaCl_2$) and later by FABMS.

The co-occurrence of sulfated and non-sulfated phenolic compounds in higher plants has been reported mainly for flavonoids.[9–20] The only sulfated anthraquinones that have so far been isolated from plants, are 'emodin-1(or 8)-monoglucoside sulfate' and emodin dianthrone diglucoside sulfate from *Rumex pulcher*,[21] and sulfemodin 8-*O*-β-D-glucoside from *Rheum emodi*.[22]

Sulfated natural products are usually isolated as their K^+ salts,[23] but other cations such as sodium and calcium have also been reported.[13,15,17,20]

Different from the above mentioned EIMS analysis, which had apparently delivered the spectrum of the sulfate-free compound, HRFABMS gave *m/z* 536.0394 (calcd for $C_{24}H_{17}O_{11}SNa$ 536.0389), while LRFABMS showed significant ions at 513 and 433 indicating the loss of sodium and sulfate portions, respectively, and a peak observed at *m/z* 80 confirmed the presence of an *O*-sulfate moiety in the sample. IR spectroscopy gave further support for the presence of a sulfate residue by showing strong bands at 1280, 1026, and 780 cm^{-1} similar to the literature values 1250 cm^{-1} (S=O), 1030 cm^{-1} (C–O–S) and 800 cm^{-1} (S–O) observed in sulfated flavonoids.[24–27] The presence of sodium was further noted from the yellow color obtained by flame assay. Furthermore, the ^{13}C NMR spectrum of the acetylphloroglucinol moiety of the phenylanthraquinone as compared to that of knipholone[8] showed downfield shift values of 7.1, 0.1, and 3.7 ppm, for carbons C-1′, C-3′, and C-5′, respectively, whilst C-6′ experienced an upfield shift of 3.6 ppm (Table 1). These carbon shifts were attributed to the presence of an *O*-sulfate group; this phenomenon had also been observed in sulfated flavonoids.[14,15,19,20,26] The shifts suggested that the sulfate group was most likely located at C-6′. This conclusion was supported by HMBC, which showed interactions of the 2′-OH proton to C-1′,

Table 1. ^{13}C NMR spectral data of the new compounds **5–8** in comparison to those of **1–4**

Compound	1	2	3	4	5	6	7	8
C-1	161.7	163.7	162.1	159.8	162.8	162.8	162.6	163.3
C-2	124.6	125.7	124.4	125.9	124.8	124.8	125.2	125.4
C-3	151.6	153.1	151.4	151.9	152.9	152.7	153.7	153.2
C-4	128.5	129.6	128.7	126.5	129.5	129.1	130.1	129.7
C-5	119.3	120.3	119.1	120.1	119.8	119.8	120.2	120.4
C-6	137.4	138.1	137.3	137.3	137.6	137.6	138.0	138.1
C-7	123.3	124.4	123.4	124.2	123.5	123.5	123.9	124.0
C-8	161.1	163.0	161.5	159.4	162.1	162.1	162.4	162.5
C-9	192.5	194.4	192.0	192.7	193.6	193.6	194.1	194.1
C-10	181.9	183.9	181.9	183.0	182.2	182.2	182.6	183.1
C-11	134.4	136.1	134.2	134.3	135.2	135.1	135.8	135.9
C-12	115.5	116.9	115.4	115.5	115.9	116.0	116.5	116.5
C-13	114.7	116.1	114.5	115.0	114.9	114.9	115.4	115.5
C-14	131.6	133.2	131.3	132.3	131.9	131.9	132.6	133.0
C-1′	104.7	110.4	104.0	103.4	111.8	113.1	110.7	117.6
C-2′	163.3	164.0	161.4	165.5	163.1	162.6	163.3	159.8
C-3′	107.3	106.9	105.8	110.0	107.2	107.6	107.1	111.2
C-4′	162.4	162.7	161.2	163.0	162.2	159.7	161.7	165.4
C-5′	91.2	95.4	94.5	100.3	94.9	98.5	99.5	105.2
C-6′	161.9	161.9	160.7	162.0	158.3	157.6	158.5	159.4
Ar-CH_3	20.4	21.0	20.4	21.4	20.9	20.9	21.5	21.7
3′-$COCH_3$	32.6	33.6	32.5	31.6	32.9	33.3	33.1	31.7
3′-$COCH_3$	202.3	205.0	202.6	203.5	204.1	204.8	204.7	204.5
2′-OCH_3	—	—	—	60.7	—	—	—	61.0
4′-OCH_3	55.6	—	—	—	55.7	—	—	—
C-1″	—	102.3	—	—	—	101.5	—	—
C-2″	—	74.8	—	—	—	74.2	—	—
C-3″	—	78.5	—	—	—	78.1	—	—
C-4″	—	78.7	—	—	—	78.3	—	—
C-5″	—	71.2	—	—	—	71.1	—	—
C-6″	—	62.6	—	—	—	62.7	—	—

C-2′, and C-3′, whilst NOESY correlations were observed between the *O*-methyl group and both, H-5′ and the methyl group of the acetyl substituent at C-3′. Therefore, the new compound was deduced to have the constitution **5** shown in Figure 1.

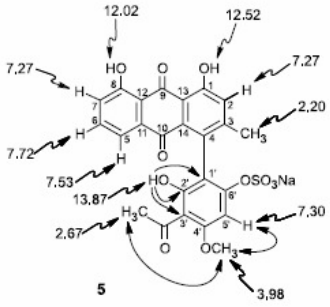


Figure 1. ^1H NMR chemical shifts as well as selected HMBC (single arrows) and NOESY (double arrows) correlations of **5**.

As with all phenylanthraquinones isolated so far, compound **5** is optically active due to the presence of a rotationally hindered biaryl axis. Its absolute configuration was established by comparison of the circular dichroism (CD) spectrum of compound **5** with that of the sulfate-free analog, knipholone (**1**). Surprisingly, the CD spectra of compounds **5** and **1** were found to be opposite to each other (Fig. 2). To exclude that this phenomenon might be due to the presence of the sulfate ester group and in order to get additional support for the constitution of **5**, the new sulfated compound was hydrolyzed (see Section 4) to yield knipholone (92%), but indeed with the opposite axial configuration (i.e., *ent*-**1**), as evidenced from its still opposite CD spectrum (Fig. 2). This proved that, as compared to **1**, compound **5** has a different stereo-orientation at the axis (although for formal reasons, it is likewise *M*-configured[7]).

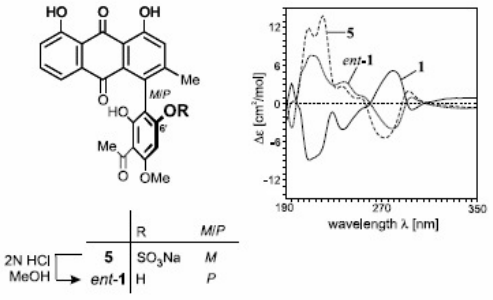


Figure 2. Comparison of the CD spectra of compound **5** and its hydrolysis product, *ent*-**1**, with the CD curve of authentic knipholone (**1**).

The FABMS (negative mode) of the second compound showed characteristic ion peaks at *m/z* 684 $[M\cdot^-]$, 661 $[M-Na]^-$, 581 $[661\text{-}SO_3]^-$, and 419 $[581\text{-Gluc}+H]^-$ corresponding to the molecular formula $C_{29}H_{25}O_{16}SNa$. Once again, as expected, the chemical shifts in the ^1H NMR (Fig. 3a) spectrum were in good agreement with those published for 4′-*O*-demethylknipholone-4′-β-D-glucopyranoside[2] except for the proton signal at H-5′, which resonated at a lower field (δ 7.51, Fig. 3a) compared to δ

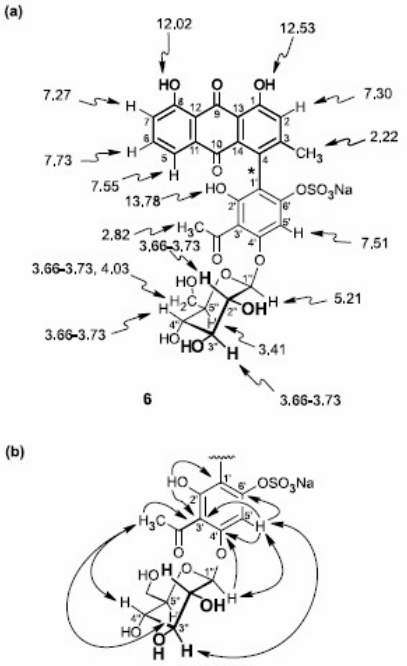


Figure 3. Diagnostically significant ^{1}H NMR data (a) as well as HMBC (single arrows) and NOESY (double arrows) interactions (b) of compound **6**.

6.38 in 4′-*O*-demethylknipholone-4′-β-D-glucopyranoside (**2**). The ^{13}C NMR data as compared to those of **2** (Table 1) showed that while carbons C-1′, C-3′ and C-5′ were shifted downfield by 2.7, 0.7, and 3.1 ppm, respectively, C-6′ underwent an upfield shift by 4.3 ppm. The chelated OH group at C-2′ revealed HMBC interactions to carbons C-1′ and C-3′; H-5′ gave correlations to C-3′, C-4′, and C-6′ (Fig. 3b). Furthermore, NOESY experiments displayed interactions between H-5′ and both, H-1″ and H-3″, whilst the methyl group of the acetyl function showed interactions to H-4″ and H-5″. The sulfate moiety was therefore, unequivocally deduced to be located on the oxygen at C-6′, so that the new compound had the constitution **6** as shown in Figure 3.

The third compound exhibited an ion peak at *m/z* 522 in the negative FABMS and its formula was deduced to be $C_{23}H_{15}O_{11}SNa$. The spectrum also showed significant ion peaks at *m/z* 499 and 419, indicative of the loss of sodium and sulfate ions, respectively. Mild acid hydrolysis of the compound afforded a product whose chromatographic and spectroscopic properties (co-TLC, co-HPLC, ^{1}H and ^{13}C NMR) were identical to those of 4′-*O*-demethylknipholone (**3**). The ^{1}H NMR spectrum displayed characteristic proton signals similar to those observed for 4′-*O*-demethylknipholone[5] except for the proton at H-5′, which resonated downfield at δ 7.20 as compared to δ 6.11 in 4′-*O*-demethylknipholone. Like for compounds **5** and **6**, the ^{13}C NMR spectrum revealed significant downfield shifts for the *ortho* carbons at C-1′ (6.7 ppm) and C-5′ (5.0 ppm), and the *para* carbon C-3′ (1.3 ppm), and an upfield shift for the *ipso* carbon C-6′ (2.2 ppm). Although, the ^{1}H NMR spectrum showed proton signals characteristic of chelated OH groups at C-2′ (13.28 ppm) and C-4′ (10.02 ppm), these did not give long-range HMBC correlations to C-1′, C-2′, C-3′, C-4′ and C-5′, which would have excluded either positions C-2′ or C-4′ to be the site of the sulfate group. However, NOESY experiments revealed interactions between the acetyl methyl and both 2′-OH and 4′-OH, whilst the proton signal at H-5′ showed correlations to 4′-OH. Thus, together with the aforementioned significant downfield and upfield shifts in ^{13}C NMR, unambiguously assigned the sulfate substituent to be located at C-6′ (see Fig. S2, Supporting information). Therefore, the new compound was deduced to have the constitution **7**.

The fourth compound was assigned the molecular formula $C_{24}H_{17}O_{11}SNa$ from negative FABMS, which displayed a molecular ion peak at *m/z* 536 [M·⁻]. The spectrum also revealed a peak at *m/z* 513 indicating the loss of the sodium ion. Acid hydrolysis yielded a compound whose chromatographic and spectroscopic data were fully identical to those observed for isoknipholone.[4] The ^{1}H NMR spectrum displayed a downfield shift of H-5′ from 6.22 in isoknipholone[4] to 7.39 in the new compound and in ^{13}C NMR, significant downfield shifts were observed for the *ortho* carbons C-1′ (14.2 ppm) and C-5′ (4.9 ppm) and for the *para* carbon C-3′ (1.2 ppm), whilst an upfield shift was found for the *ipso* carbon C-6′ (2.6 ppm). HMBC experiments indicated correlations between the hydroxyl group at C-4′ and C-3′, C-4′, and C-5′, whereas the *O*-methyl group showed correlation to C-2′. Furthermore, NOESY measurements displayed interactions of the methoxy group to both the aryl methyl group (at C-3) and the aryl acetyl (at C-3′). The above data suggested that the sulfate moiety was located at position C-6′ (see Fig. S3, Supporting information) and thus, the new compound had the structure **8**.

Compounds **5**–**8** were all isolated and identified as sodium salts. They were observed to be highly polar, with R_f values of 0.08, 0.05, and 0.06 in $CHCl_3$/MeOH (9:1) for **5**, **7**, and **8**, and of 0.1 in $CHCl_3$/MeOH (4:1) for **6**, as compared to their sulfate-free analogs, which had R_f values of 0.8, 0.5, 0.7, and 0.6 for **1**, **3**, **4**, and **2**, respectively.

Compounds **6**–**8** were all found to be optically active due to the presence of the rotationally hindered biaryl axis and were thus, configurationally stable. Their absolute configurations were determined, as already described for **5**, by CD comparison with their sulfate-free analogs **2**, **3**, and **4**, respectively (see Fig. S4, Supporting information). Different from **5** (whose axial configuration was opposite to that of knipholone, **1**, see Fig. 2), **6**–**8** all had the same stereo-orientation as **2**–**4**.

A solution of sodium 4′-*O*-demethylknipholone 6′-*O*-sulfate (**7**) in acetone slowly generated crystals of quality sufficient for an X-ray structure analysis, a lucky circumstance because no crystal structure analysis had so far succeeded in the field of phenylanthraquinones. This crystal structure analysis gave rise to two surprises: firstly, the compound that had crystallized out was not compound **7**,

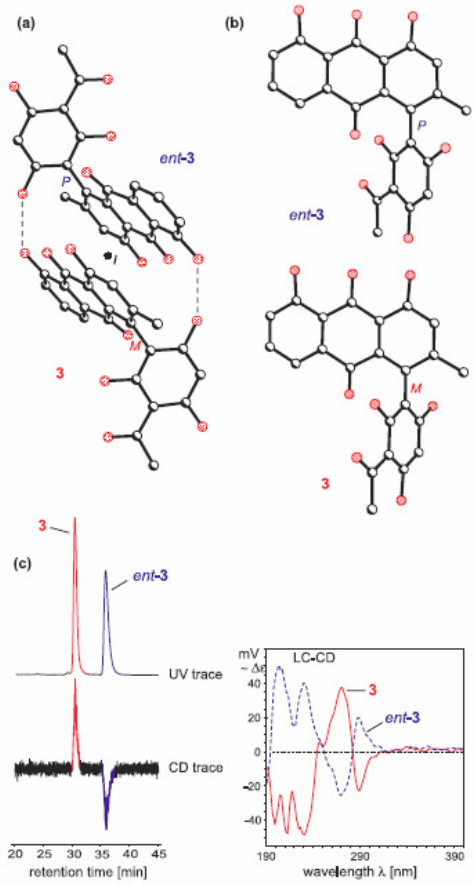


Figure 4. Racemic 4′-*O*-demethylknipholone (*rac*-**3**): crystal structure showing, both, the *M*- and the *P*-enantiomers, **3** and *ent*-**3**, in their relative orientation to each other in the crystal (a) and 'isolated' from each other, for reasons of clarity (the solvent molecule, acetone, has been omitted) (b); enantiomeric resolution on a chiral HPLC phase (Chiracel OD-H) with UV and CD detection (both monitored at 277 nm), and full CD spectra of the two enantiomers of **3** measured online, right from the peaks (c); *i*=centre of inversion.

but the sulfate-free 'parent compound', 4′-*O*-demethylknipholone (**3**, see Fig. 4), indicating that even under the mild conditions of crystallization, sulfate hydrolysis had taken place and that this slow hydrolysis was apparently a good precondition for the formation of suitable crystals. It is generally known that sulfate ester bonds are highly labile and among flavonoids it has been observed that some compounds spontaneously hydrolyze upon standing in methanol.[14,15] The second surprise was that **3** had crystallized in a racemic form, although, as described above, compound **7** had been optically active and had shown a significant CD spectrum, indicating that possibly the natural product **7** had not been enantiomerically pure, but only enantiomer-enriched. Even more remarkably, in the case of knipholone itself the *M*-enantiomer **1** prevails for the isolated sulfate-free compound and the other enantiomer for the sulfate **5** (for formal reasons likewise to be denoted as *M*[7]); the large portion of racemic material in the case of **7** had now—besides the slow formation of the product (see above)—been another favorable circumstance for the improved crystallization properties of the hydrolyzed material, **3**.

The presence of racemic or near-racemic material warranted the availability of an analytical method for the determination of the enantiomeric ratios of all these natural products. This was achieved by chromatography on a chiral OD-H phase (see Section 4), indeed showing the crystalline material to be fully racemic (Fig. 4, exact values measured experimentally: 51.3% *M* and 48.7% *P*), while the mother liquor displayed a slight enrichment (54.1:45.9) in favor of the *M*-enantiomer.

The enantiomer analysis on the chiral phase then also proved to be applicable to the resolution of the other isolated phenylanthraquinones, showing that indeed only two of them were atropisomerically pure [4′-*O*-demethylknipholone-4′-β-D-glucopyranoside, **2** (95:5)] or nearly pure [4′-*O*-demethylknipholone, **3** (93:7)], while knipholone (**1**) proved to be a 60:40 *M* to *P* mixture and isoknipholone (**4**) was even virtually racemic (52:48, see Table 2).

Exemplarily for knipholone, the analytical resolution of the naturally occurring *M* to *P* mixture on the chiral phase was successfully extended to a semi-preparative scale, to give enantiomerically pure *M* (1.2 mg; 100:0, *M* to *P*) and a *P*-rich fraction (0.5 mg; 6:94, *M* to *P*). The pure *M*-knipholone gave an optical rotation $[\alpha]_D^{20}$ +81 (*c* 0.09, MeOH) almost identical to the value initially reported for knipholone, $[\alpha]_D^{20}$ +80 (*c* 0.01, MeOH), by Dagne and Steglich.[8] With this first $[\alpha]_D$ of a provenly enantiomerically pure sample of *M*-knipholone, determined as +81, it was then possible to deduce the enantiomeric composition of the above *M* to *P* mixture from the measured optical rotation of +22 (*c* 0.04, MeOH), to be 64:36, which is reasonably close to the chromatographically measured value of 60:40. Similarly, the enantiomerically nearly pure *P* isolated (6:94, *M* to *P*) was observed to have $[\alpha]_D^{20}$ −59 (*c* 0.03, MeOH; calcd er.: 13:87 *M* to *P*).[28] For compounds **2**–**4**, the sulfates **6**–**8** and their respective hydrolysis products, however, the $[\alpha]_D$ values were not as reliable and reproducible as those obtained for knipholone—a problem typical of strongly colored compounds,[29] showing once again the value of now having the chromatography on a chiral phase available for the analysis of enantiomeric ratios.

Unfortunately, the sulfates **5**–**8** themselves were not amenable to HPLC analysis on a chiral phase due to their highly polar nature so that they could be analyzed only via their hydrolyzed products (see Table 2). Note, however, that possibly some or even all the sulfates, **5**–**8**, lose their enantiomeric purities during hydrolysis (either by spontaneous hydrolysis during isolation or chemically using 2 N HCl). This becomes evident for the sulfated glycoside **6**: since in this case, due to the presence of the chiral glucose portion, the rotational isomers are diastereomers, NMR clearly reveals that the hydrolysis product of **6**, viz.

Table 2. Results of the enantiomeric analysis of phenylanthraquinones

Compound	Enantiomeric ratios	
	M (%)	*P* (%)
Knipholone (**1**)	60	40
4′-*O*-Demethylknipholone-4′-*O*-β-D-glucopyranoside (**2**)	95	5
4′-*O*-Demethylknipholone (**3**)	93	7
Isoknipholone (**4**)	52	48
ent-Knipholone[a]	42	58
4′-*O*-Demethylknipholone-4′-*O*-β-D-glucopyranoside[a]	58	42
4′-*O*-Demethylknipholone[a]	51	49
Isoknipholone[a]	52	48

[a] Obtained by acid hydrolysis of the respective sulfates, **5**–**8**.

compound **2**, is a near 1:1 mixture of atropisomers (see Table 2 and Supporting information) consistent with the chiral phase analysis, whereas the respective sulfate precursor, compound **6**, and also the genuine, isolated **2** are clearly one species each, according to ^{1}H NMR (see Supporting information).

The most remarkable stereochemical relationship is that between knipholone (**1**) and its sulfate, **5**: although, constitutionally, they only differ by the (easily hydrolyzable) sulfate group, they belong to (preferentially) opposite stereochemical series, so that one has to expect that the (as such nearly enantiomerically pure) sulfate **5** upon hydrolysis would always diminish the enantiomeric purity of the (opposite-configured) 'parent' knipholone—to the best of our knowledge a unique case in natural product chemistry.

For a further confirmation of structure, one of the novel phenylanthraquinone sulfates was synthesized from its sulfate-free analog (Fig. 5). Thus, reaction of *ent*-**1** with tetrabutylammonium hydrogensulfate (TBAHS, 4 equiv) and dicyclohexylcarbodiimide (DCC, 20 equiv) in pyridine at 80 °C (15 min),[30] followed by cation exchange to give the sodium salt, yielded sodium *ent*-knipholone 6′-*O*-sulfate (**5**, 61%). Since knipholone itself had already been prepared by an atropo-enantiodivergent total synthesis,[31] the partial synthesis of its 6′-*O*-sulfate simultaneously constitutes a formal total synthesis of the sulfated natural product, **5**.

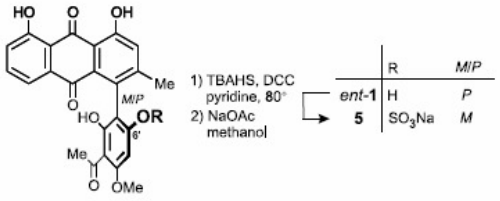


Figure 5. Reaction conditions for the partial synthesis of the sulfate **5**.

Due to the in part high antimalarial activities of knipholone and its analogs,[3] the new isolated phenylanthraquinones **5**–**8** were submitted to in vitro antiplasmodial tests against the chloroquine resistant strain K1 of *Plasmodium falciparum*. Compound **7** showed weak activity, while the other three were not active even at the highest concentration tested (5.0 μg/mL) (Table 3). The sulfate-free compound isoknipholone (**4**), however, which was tested for the first time in this paper, was found to have considerable antiplasmodial activity in vitro, comparable to that of knipholone anthrone,[3] thus, being the compound with the as yet highest activity among the phenylanthraquinones previously tested (Table 3). This made it interesting to examine its cytotoxic effects on mammalian cells, which were fortunately found to be much weaker, by a factor of >360. None of the above compounds showed any activity in vitro against *Trypanosoma brucei rhodesiense* (causes African sleeping sickness) or *Trypanosoma cruzi* (pathogen of the South American Chagas' disease).

3. Conclusion

From the polar fraction of *B. frutescens* roots, the first *O*-sulfated phenylanthraquinones have been discovered, occurring as the respective sodium salt with the sulfate substituent at the 6′-position of the acetyl phloroglucinol moiety. Chiroptical analysis of these four new compounds revealed that three of them, viz. sodium 4′-*O*-demethylknipholone-4′-β-D-glucopyranoside 6′-*O*-sulfate (**6**),

Table 3. Biological activities of **4**–**8** against *P. falciparum* (K1 strain), *T. brucei rhodesiense*, and *T. cruzi* and cytotoxicities against rat skeletal myoblast cells (L6)

Compound	IC_{50} (μg/mL)			
	P. falciparum	*T. cruzi*	*T. brucei rhodesiense*	*Cytotoxicity*
Standard	0.0461[a]	0.26[b]	0.0029[c]	nd[d]
4	0.12	14.4	9.94	43.5
5	>5	>90	56.3	nd[d]
6	>5	>90	25.5	nd[d]
7	4.13	>90	56.7	nd[d]
8	>5	>90	53.8	nd[d]

[a] Chloroquine.
[b] Benznidazole.
[c] Melarsoprol.
[d] Not determined.

sodium 4′-*O*-demethylknipholone 6′-*O*-sulfate (**7**), and sodium isoknipholone 6′-*O*-sulfate (**8**), exhibited the same main stereo-orientation at the biaryl axis as their sulfate-free analogs, **2**–**4**. The configurations of sodium *ent*-knipholone 6′-*O*-sulfate (**5**) and its hydrolysis product, *ent*-knipholone, by contrast, were observed to be opposite to that of knipholone (**1**). Enantiomeric analysis of compounds **1**–**4** and the hydrolysis products of **5**–**8**, achieved successfully by chromatography on a chiral OD-H HPLC phase, indicated that all these compounds were not enantiomerically pure. For the first time in the field of natural phenylanthraquinones, an X-ray structure analysis is reported in this paper, viz. for 4′-*O*-demethylknipholone (**3**). The sulfated phenylanthraquinones **5**–**8** showed no significant activity against the test organisms, *P. falciparum*, *T. cruzi*, and *T. brucei. rhodesiense*, while the sulfate-free compound isoknipholone (**4**) was found to possess a good antiplasmodial in vitro activity with essentially no cytotoxicity.

4. Experimental

4.1. General

Melting points were determined on a Griffin melting point instrument and are uncorrected. Optical rotations were measured on a JASCO P-1030 polarimeter using a thermostated cell (20 °C, 10-cm cell). UV–vis spectra were performed on a Shimadzu UV-2101PC spectrometer. CD spectra were recorded on a J-715 spectropolarimeter (JASCO Deutschland, Gross-Umstadt, Germany) at room temperature using a 0.05-cm standard cell and spectrophotometric grade MeOH, and are reported in $\Delta\varepsilon$ in cm^2/mol at the given wavelength λ (nm). Stereoanalytical separations were carried out on a chiral stationary phase employing a Chiralcel OD-H HPLC column (dimensions: 4.6×250 mm; particle size: 5 μm) from Daicel Chemical Industries Ltd (Tokyo, Japan). For HPLC-CD coupling experiments, the J-715 CD spectrometer was equipped with a PU-1580 pump (JASCO), a LG-980-02S ternary gradient unit, a 7725i Rheodyne injector valve, an ERC-7215 UV detector hyphenated to a J-715 spectropolarimeter with a 5 mm standard flow cell, and the Borwin chromatographic software (JASCO Deutschland). IR spectra were carried out on a Perkin-Elmer 2000 FT-IR spectrometer. ^1H and ^{13}C NMR were obtained on a Bruker Avance 300, 400 and DMX 600 (300, 400 and 600 MHz) instruments using CD_3COCD_3 as the solvent. FABMS was carried out, in the negative mode, with a Finnigan MAT 90 instrument using 3-nitrobenzylalcohol as the matrix. Flash chromatography was carried out using columns packed with silica gel 60 (particle size 0.040–0.063 mm). Gel filtration was achieved using Sephadex LH-20. Preparative TLC plates (0.5 mm thick) were prepared using either silica gel 60 $HF_{254+366}$ (Merck, Germany) or silica gel 60 PF_{254} containing $CaSO_4$ (Merck, Germany) on 20×20 cm glass plates. Spots were detected under UV light.

4.2. Plant material

The roots of *B. frutescens* were harvested from the chemistry experimental garden at the University of Botswana in September 2003. Voucher specimens have been deposited at the Herbarium, Biological Sciences, University of Botswana (code BA 205) and at the Herbarium Bringmann, University of Würzburg (code 60).

4.3. Extraction and isolation

Dried powdered roots (1.8 kg) were soaked in CH_2Cl_2/MeOH (1:1) for 24 h followed by MeOH for 30 min. The two extracts, combined and freed of solvent, yielded an organic extract (129 g), which was subjected to flash chromatography eluted first with petroleum ether and then with increasing amounts of ethyl acetate. Fractions (about 250 mL each) were collected as follows: fraction 1 (100% petroleum ether), fractions 2–4 (20% EtOAc), fraction 5 (50% EtOAc), fraction 6 (100% EtOAc), fraction 7 (10% MeOH/EtOAc), fraction 8 (20% MeOH/EtOAc). Fractions 6–8 were combined since they contained the same compounds according to TLC.

Fractions 6–8 (33 g) were applied to Sephadex LH-20 ($CHCl_3$/MeOH, 1:1) giving six fractions, of which fractions 3–6 were submitted to PTLC ($CHCl_3$/MeOH, 4:1) each yielding four bands. Repeated chromatography on Sephadex LH-20 ($CHCl_3$/MeOH, 2:1) of bands 1 and 2 afforded sodium 4′-*O*-demethylknipholone-4′-β-D-glucopyranoside 6′-*O*-sulfate (**6**, 22 mg) and sodium 4′-*O*-demethylknipholone 6′-*O*-sulfate (**7**, 43 mg). However, in most cases bands 3 and 4 were not well resolved so they were combined and subjected to PTLC (EtOAc/MeOH, 2:1) giving sodium *ent*-knipholone 6′-*O*-sulfate (**5**, 21 mg) and sodium isoknipholone 6′-*O*-sulfate (**8**, 5 mg).

A portion of fraction 5 (500 mg) was subjected to chromatography on Sephadex LH-20 ($CHCl_3$/MeOH, 2:1) followed by PTLC ($CHCl_3$/MeOH, 95:5) giving 4′-*O*-demethylknipholone-4′-β-D-glucopyranoside (**2**, 8 mg; $[\alpha]_D^{20}$ +8 (*c* 0.04, MeOH); 95:5 *M* to *P*; lit.[2] $[\alpha]_D^{25}$ −218; note: the value has apparently been a mistake; the sample has been remeasured, showing $[\alpha]_D^{20}$ +36, (*c* 0.06, MeOH)) and 4′-*O*-demethylknipholone (**3**, 4 mg; $[\alpha]_D^{20}$ +113 (*c* 0.04, MeOH); 93:7 *M* to *P*; lit.[5] $[\alpha]_D$ +104). Like in the case of their sulfated analogs, knipholone and isoknipholone were isolated as a mixture, which was successfully separated by PTLC (*n*-hexane/acetone, 2:1) yielding knipholone (**1**, 95 mg; $[\alpha]_D^{20}$ +22 (*c* 0.04, MeOH); 60:40 *M* to *P*; lit.[8] $[\alpha]_D^{22}$ +80) and isoknipholone (**4**, 11 mg; $[\alpha]_D^{20}$ −12 (*c* 0.06, MeOH); 52:48 *M* to *P*; lit.[4] $[\alpha]_D^{24}$ +33.3).

4.3.1. Sodium *ent*-knipholone 6′-*O*-sulfate (5). Red amorphous powder: mp 182–184 °C (dec); $[\alpha]_D^{20}$ −4 (*c* 0.05, MeOH); UV–vis (MeOH): λ_{max} 431 (log ε 4.22), 343 (log ε 3.96), 285 (log ε 4.62), 256 (log ε 4.64), 225 (log ε 4.85); CD (MeOH): $\Delta\varepsilon_{209}$+11.9, $\Delta\varepsilon_{221}$+13.8, $\Delta\varepsilon_{237}$+2.7, $\Delta\varepsilon_{252}$+0.8, $\Delta\varepsilon_{273}$−5.4, $\Delta\varepsilon_{293}$+1.9; IR (KBr): ν_{max} 3541, 3475, 3414, 2924, 1616, 1460, 1372, 1280, 1107, 1026, 780 cm^{-1}; ^1H NMR (300 MHz, CD_3COCD_3): δ 2.20 (s, 3H, CH_3-3), 2.67 (s, 3H, CH_3CO), 3.98 (s, 3H, CH_3O), 7.27 (s, 1H, H-2), 7.27 (dd, *J*=8.3, 1.2 Hz, 1H, H-7), 7.30 (s, 1H, H-5′), 7.53 (dd, *J*=7.6, 1.1 Hz, 1H, H-5), 7.72 (dd, *J*=8.2, 7.7 Hz, 1H, H-6), 12.02 (s, 1H, 8-OH), 12.52 (s, 1H, 1-OH), 13.87 (s, 1H, 2′-OH); ^{13}C NMR (150 MHz, CD_3COCD_3): δ, see Table 1; MS (EI): *m*/*z* (%)=434 (100) [M−$NaSO_3$+H],

419 (66); MS (FAB): *m/z* (%)=536 (32) [M·$^-$], 513 (100) [M−Na]$^-$, 433 (40) [513-SO_3]$^-$, 80 (7) [SO_3]$^-$; MS (FAB) exact mass calcd for $C_{24}H_{17}O_{11}SNa$: 536.0389; found 536.0394.

4.3.2. Sodium 4′-*O*-demethylknipholone-4′-β-D-glucopyranoside 6′-*O*-sulfate (6). Orange-red colored amorphous powder: mp 201–203 °C (dec); $[\alpha]_D^{20}$ −16 (*c* 0.05, MeOH); UV–vis (MeOH): λ_{max} 433 (log ε 3.41), 340 (log ε 3.20), 279 (log ε 3.83), 256 (log ε 3.86), 220 (log ε 4.01); CD (MeOH): $\Delta\varepsilon_{195}$+1.2, $\Delta\varepsilon_{209}$−16.2, $\Delta\varepsilon_{221}$−18.5, $\Delta\varepsilon_{296}$+7.8, $\Delta\varepsilon_{292}$−3.1; IR (KBr): ν_{max} 3418, 1622, 1383, 1279, 1246, 1076, 1050, 1026, 776, 611 cm^{-1}; ^{1}H NMR (600 MHz, CD_3COCD_3): δ 2.22 (s, 3H, CH_3-3), 2.82 (s, 3H, CH_3CO), 3.41 (m, 1H, H-5″), 3.66–3.73 (m, 4H, CH_2OH), 4.03 (m, 1H, CH_2OH), 5.21 (d, *J*=7.4 Hz, 1H, H-1″), 7.27 (dd, *J*=8.3 Hz, 1H, H-7), 7.30 (s, 1H, H-2), 7.51 (s, 1H, H-5′), 7.55 (dd, *J*=7.5 Hz, 1H, H-5), 7.73 (t, *J*=8.0 Hz, 1H, H-6), 12.02 (s, 1H, 8-OH), 12.53 (s, 1H, 1-OH), 13.78 (s, 1H, 2′-OH); ^{13}C NMR (150 MHz, CD_3COCD_3): δ, see Table 1; MS (FAB): *m/z* (%)=684 (6) [M·$^-$], 661 (13) [M−Na]$^-$, 581 (4) [661-SO_3]$^-$, 419 (4) [581-Gluc+H]$^-$; MS (FAB) exact mass calcd for $C_{29}H_{25}O_{16}SNa$: 684.0758; found 684.0769.

4.3.3. Sodium 4′-*O*-demethylknipholone 6′-*O*-sulfate (7). Red amorphous powder: mp 161–163 °C (dec); $[\alpha]_D^{20}$ +67 (*c* 0.06, MeOH); UV–vis (MeOH): λ_{max} 430 (log ε 4.34), 288 (log ε 4.75), 255 (log ε 4.74), 225 (log ε 4.96); CD (MeOH): $\Delta\varepsilon_{220}$−27.4, $\Delta\varepsilon_{270}$+13.5, $\Delta\varepsilon_{292}$−3.6; IR (KBr): ν_{max} 3549, 3467, 3416, 2918, 1623, 1458, 1425, 1367, 1280, 1086, 1048, 1022, 781 cm^{-1}; ^{1}H NMR (600 MHz, CD_3COCD_3): δ 2.23 (s, 3H, CH_3-3), 2.69 (s, 3H, CH_3CO), 7.20 (s, 1H, H-5′), 7.28 (d, *J*=6.6 Hz, 1H, H-7), 7.29 (s, 1H, H-2), 7.56 (dd, *J*=7.5, 0.5 Hz, 1H, H-5), 7.74 (d, *J*=8.0 Hz, 1H, H-6), 10.02 (s, 1H, 4′-OH), 12.03 (s, 1H, 8-OH), 12.53 (s, 1H, 1-OH), 13.28 (s, 1H, 2′-OH); ^{13}C NMR (150 MHz, CD_3COCD_3): δ, see Table 1; MS (FAB): *m/z* (%)=522 (31) [M·$^-$], 499 (85) [M−Na]$^-$, 419 (41) [499-SO_3]$^-$; MS (FAB) exact mass calcd for $C_{23}H_{15}O_{11}SNa$: 522.0231; found 522.0227.

4.3.4. Sodium isoknipholone 6′-*O*-sulfate (8). Red amorphous powder: mp 105–108 °C (dec); $[\alpha]_D^{20}$ −27 (*c* 0.03, MeOH); UV–vis (MeOH): λ_{max} 427 (log ε 3.26), 338 (log ε 3.00), 285 (log ε 3.68), 255 (log ε 3.83), 205 (log ε 4.02); CD (MeOH): $\Delta\varepsilon_{215}$−1.8, $\Delta\varepsilon_{251}$+2.4, $\Delta\varepsilon_{294}$−0.3; IR (KBr): ν_{max} 3541, 3467, 3416, 2923, 1618, 1417, 1384, 1265, 1107, 1026, 622 cm^{-1}; ^{1}H NMR (600 MHz, CD_3COCD_3): δ 2.26 (s, 3H, CH_3-3), 2.65 (s, 3H, CH_3CO), 3.39 (s, 3H, CH_3O), 7.30 (dd, *J*=8.4, 1.1 Hz, 1H, H-7), 7.32 (s, 1H, H-2), 7. 39 (s, 1H, H-5′), 7.58 (dd, *J*=7.6 Hz, 1H, H-5), 7.77 (dd, *J*=8.3, 7.5 Hz, 1H, H-6), 13.25 (s, 1H, 4′-OH); ^{13}C NMR (150 MHz, CD_3COCD_3): δ, see Table 1; MS (FAB): *m/z* (%)=536 (5) [M·$^-$], 513 (23) [M−Na]$^-$; MS (FAB) exact mass calcd for $C_{24}H_{17}O_{11}SNa$: 536.0389; found 536.0394.

4.4. Energy dispersive X-ray (EDX) analysis

The white inorganic substance originating in the hydrolysis of phenylanthraquinone sulfates was analyzed by using an environmental scanning electron microscope (ESEM) fitted with an EDX analysis system (EDAX 3.10 with an ultra thin window). The sample, mounted on aluminum pin type mounts (3 mm) using a carbon tape, was examined under low vacuum using a Phillips XL 30 ESEM microscope fitted with a tungsten filament and a gaseous secondary electron detector.

4.5. Acid hydrolysis

Compounds **5**–**8** (1.1, 1.2, 1.3, and 1.0 mg, respectively) were dissolved in MeOH (2 mL each) and mixed with 2 N HCl (5 mL). The mixture was stirred at room temperature for 30 min, after which the sulfate-free phenylanthraquinones were extracted with EtOAc (3×5 mL), purified on a small Sephadex LH-20 column ($CHCl_3$/MeOH, 2:1), and analyzed by TLC and ^{1}H NMR: *ent*-knipholone (*ent*-**1**, 0.8 mg, 1.8 μmol, 92%), 4′-*O*-demethylknipholone (**3**, 0.9 mg, 2.1 μmol, 90%), isoknipholone (**4**, 0.7 mg, 1.6 μmol, 90%). Acid hydrolysis of sodium 4′-*O*-demethylknipholone-4′-β-D-glucopyranoside 6′-*O*-sulfate (**6**), subsequent extraction with EtOAc (8×5 mL), and TLC indicated the presence of two compounds, which were separated on a small silica gel column (SiO_2, $CHCl_3$/MeOH, 96:4 then 90:10) delivering 4′-*O*-demethylknipholone (**3**, 0.2 mg, 0.48 μmol, 36%) and 4′-*O*-demethylknipholone-4′-β-D-glucopyranoside (**2**, 0.5 mg, 0.86 μmol, 51%), the latter being identical to the authentic compound **2** except for the peak doubling in NMR (see Fig. S2, Supporting information) showing the presence of diastereomers, as evident also by analysis on a chiral phase (Table 2). The aqueous layer was concentrated and tested with $BaCl_2$.

4.6. Resolution of atropisomers

Solutions of compounds **1**, **2**, **3**, and **4** (0.5 mg each) in MeOH (500 μL each) were resolved by HPLC on a Chiralcel OD-H column (4.6 mm×250 mm, 5 μm), with an isopropanol/*n*-hexane gradient under the following conditions: flow 0.5 mL/min; 0–10 min: isopropanol/*n*-hexane 10:90, 10–40 min: isopropanol/*n*-hexane 50:50, giving the following retention times: for **1** [t_R=31.5 min for (*M*)-**1**, t_R=33.9 min for (*P*)-**1**], for **2** [t_R=36.2 min for (*M*)-**2**, t_R=34.4 min for (*P*)-**2**], for **3** [t_R=35.5 min for (*M*)-**3**, t_R=42.4 min for (*P*)-**3**], for **4** [t_R=20.4 min for (*M*)-**4**, t_R=24.1 min for (*P*)-**4**]; the peaks were analyzed stereochemically by HPLC–CD coupling.

4.7. Partial synthesis of sodium *ent*-knipholone 6′-*O*-sulfate (5)

A solution of *ent*-**1** (5.0 mg, 11.5 μmol) in pyridine (2 mL) was treated under nitrogen with TBAHS (15.6 mg, 45 μmol) and DCC (47.5 mg, 230 μmol) and stirred at 80 °C for 15 min. After cooling to room temperature, pyridine was removed by repeated addition of toluene and evaporation of the solvent mixture. The reaction medium was diluted with MeOH (2 mL) and filtered several times through Celite to remove the dicyclohexylurea precipitate. Conversion of the TBA salt to the respective sodium salt was performed by addition of a saturated solution of NaOAc (2 mL) in MeOH, after which the mixture was purified by repeated PTLC ($CHCl_3$/MeOH, 4:1) giving sodium *ent*-knipholone 6′-*O*-sulfate (**5**, 3.6 mg, 6.7 μmol, 61%). The synthetic product

proved to be identical in all respects with natural sodium *ent*-knipholone 6′-*O*-sulfate (**5**).

4.8. Biological experiments

Antiparasitic activities against the pathogens *P. falciparum*, *T. cruzi*, and *T. brucei rhodesiense*, as well as cytotoxicities (rat skeletal myoblast L-6 cells) were assessed as described earlier.[32]

4.9. X-ray crystallographic data

The X-ray data were collected from shock-cooled, oil coated crystals on a BRUKER SMART-APEX diffractometer with a D8-goniometer equipped with a low-temperature device in ω-scan mode at 173(2) K,[33] using graphite-monochromated Mo K_α radiation ($\lambda=0.71073$ Å). The structure was integrated with SAINT[34] and a semiempirical absorption correction (SADABS)[35] was applied. The structure was solved by direct methods (SHELXS97)[36] and refined by full-matrix least-squares methods against F^2 (SHELXL97).[37]

Crystallographic data (excluding structure factors) for the structure in this paper has been deposited with the Cambridge Crystallographic Data Centre as supplementary publication number CCDC 242760. Copies of the data can be obtained, free of charge, on application to CCDC, 12 Union Road, Cambridge CB2 1EZ, UK [fax: +44 1223 336033 or e-mail: deposit@ccdc.cam.ac.uk].

Acknowledgements

Financial support by the Deutsche Forschungsgemeinschaft (Graduiertenkolleg 'Elektronendichte', to G. B.), the Fonds der Chemischen Industrie (to G. B.), and the UNDP/World Bank/WHO Special Programme (to G. B. and R. B.) is gratefully acknowledged. J. M. and M. B. are grateful for fellowships from DAAD—NAPRECA (Deutscher Akademischer Austauschdienst—Natural Products Research Network for Eastern and Central Africa). Particular thanks are due to Elke Gobright and Christian Scheurer for carrying out the in vitro assays, Prof. Dr. Dietmar Stalke for help with the X-ray structure analysis, and to JASCO company, Dr. Stephan Wagner and Jürgen Daiβ for their assistance with optical rotation measurements. B. M. A. acknowledges the University of Botswana research grant administered through the Faculty Research and Publications Committee.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tet.2005.06.055

Copies of the ^{1}H and ^{13}C NMR spectra for **5**, **6**, **7**, and **8**. ^{1}H NMR spectra for **2** (as isolated from the plant and as obtained by acid hydrolysis of the respective sulfate **6**), and synthetic **5**. EDX graph of white precipitate. ^{1}H NMR shifts as well as HMBC and NOESY correlations of compounds **7** and **8**. CD spectra of compounds **6**–**8** in comparison to their sulfate-free analogs, **2**–**4**.

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